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Short communication

Isolation and characterization of bifidobacteria from ovine cheese

Vera Bunesova [a,*], Jiri Killer [a,b], Eva Vlkova [a], Sarka Musilova [a], Martin Tomaska [c], Vojtech Rada [a], Vladimir Kmet [d]

[a] Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamycka 129, Prague 6-Suchdol 165 21, Czech Republic
[b] Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 4-Krč 14 200, Czech Republic
[c] Dairy Research Institute, Dlhá 95, 010 01 Žilina, Slovakia
[d] Institute of Animal Physiology of Slovak Academy of Sciences, Soltesovej 4, 040 01 Kosice, Slovakia

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ABSTRACT

Animal products are one of the niches of bifidobacteria, a fact probably attributable to secondary contamination. In this study, 2 species of the genus *Bifidobacterium* were isolated by culture-dependent methods from ovine cheeses that were made from unpasteurized milk without addition of starter cultures. The isolates were identified as *Bifidobacterium crudilactis* and *Bifidobacterium animalis* subsp. *lactis* using matrix-assisted laser desorption/ionization time-of-flight analysis and sequencing of phylogenetic markers (16S rRNA, *hsp60*, and *fusA*).

1. Introduction

The most common genera of lactic acid bacteria in ovine milk include *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Enterococcus* (Chebeňová-Turcovská et al., 2011; Pangallo et al., 2014). Strains identified in ovine cheese and bryndza cheese by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis belonged to 10 species of non-enterococcal lactic acid bacteria, i.e. *Lactobacillus casei*/*Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus brevis*, *Lactococcus lactis*, *Pediococcus pentosaceus*, and *Pediococcus acidilactici* (Kmet and Drugdova, 2012). There are no data for the occurrence of bifidobacteria in ovine cheese, except for the identification of the family *Bifidobacteriaceae* by pyrosequencing (Alegría et al., 2012). Bifidobacteria usually require an anaerobic environment and neutral pH to survive and maintain their numbers. Cheese, however, does provide an environment that would be conducive to the long-term survival of bifidobacteria (Boylston et al., 2004). The aim of our work was to detect bifidobacteria in ovine cheese using cultivation techniques and to identify and characterize them.

2. Material and methods

2.1. Origin of ovine cheese

Ovine lump cheeses were made in May 2013 by the traditional way in Slovak chalets (Table 1) from raw sheep milk without any thermal treatment and without using any starter cultures; fermentation was carried out only with the naturally occurring microflora. The cheeses were semi-soft and short-time ripened (3–6 days at the temperature from 20 °C to 26 °C). After that they were delivered to a dairy for further processing. The typical dry matter of cheeses was varied between 48.8 g/100 g and 52.9 g/100 g, the fat content was from 24.5 g/100 g to 30.5 g/100 g, and the pH values were from 4.9–5.2. The samples were collected by an educated sampler and analyzed for the presence of cow's and goat's milk (IC-Bovino and IC-Caprino, Zeu Inmunotec, Spain) prior to other analyses. All samples contained only sheep milk.

2.2. Cultivation and isolation of bifidobacteria

Ten grams of each tested cheese were aseptically homogenized in 90 mL of sterile saline peptone diluent (Oxoid) and serially diluted under anaerobic conditions. Appropriate dilutions were transferred to sterile dishes and immediately filled with Wilkins-Chalgren agar (WSPmup) supplemented with soya peptone (5 g/L, Oxoid), L-cysteine (0.5 g/L, Sigma), Tween 80 (1 mL/L, Sigma), mupirocin (100 mg/L, Merck), and glacial acetic acid (1 mL/L). The dishes were cultivated in

* Corresponding author. Tel.: +420 2 24 38 26 83; fax: +420 2 24 38 27 60.
E-mail address: bunesova@af.czu.cz (V. Bunesova).

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Table 1
The origin of tested bifidobacterial strains.

Sample	Identified as	Origin		Country
		Village (chalet)	Region	
S1	*B. animalis* subsp. *lactis*	Štvrtok	Spiš	Slovakia
S7[a]	*B. animalis* subsp. *lactis*	Stará Ľubovňa	Spiš	
S10[a]	*B. crudilactis*	Odorín	Spiš	
S11	*B. crudilactis*	Iliašovce	Spiš	
S13[a]	*B. crudilactis*	Lipany	Hornotoryšský, Spiš	
S15	*B. crudilactis*	Jakubovany	Šariš	
S17[a]	*B. crudilactis*	Vrchteplá	Považie	
S18	*B. crudilactis*	Slopná	Považie	
BLAC	*B. animalis* subsp. *lactis*	Type strain	DSM 10104	
BCRU	*B. crudilactis*	Type strain	LMG 23609	

DSM — Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; LMG — Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium.
[a] The partial 16S rRNA gene sequences of selected strains are available at the GenBank database (the accession numbers in text).

anaerobic jars (Oxoid, HP11A) under a CO_2/H_2 (20/80) atmosphere at 37 °C for 3 days. Pure isolates were enriched in Wilkins-Chalgren broth supplemented with soya peptone (5 g/L, Oxoid) and identified as members of the genus *Bifidobacterium* by their morphology, Gram staining, and demonstration of fructose-6-phosphate phosphoketolase activity (F6PPK-test) (Orban and Patterson, 2000). Strains from the culture collections *Bifidobacterium animalis* subsp. *lactis* DSM 10104 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and *Bifidobacterium crudilactis* LMG 23609 (Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium) were used as controls.

2.3. MALDI-TOF

For the bacterial analysis of each isolate by MALDI-TOF, the cells of fresh overnight cultures were used to prepare the samples according to the microorganism profiling ethanol–formic acid extraction procedure. Each sample spot was overlaid with 2 μL of matrix solution (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid) and again air-dried for 15 min (Bruker Daltonik GmbH, Germany). To identify the microorganisms, the raw spectra obtained for each isolate were imported into the BioTyper software, version 2.0 (Bruker Daltonik) and analyzed without any user intervention.

2.4. Phenotypic and fingerprint characteristics

Fermentation characteristics and enzymatic test results of the isolates were obtained using API 50CHL and Rapid ID32A kits (BioMérieux). The ability of the strains to grow at 46 °C was tested by culturing in Wilkins-Chalgren broth supplemented with soya peptone (5 g/L, Oxoid) for 48 h (Delcenserie et al., 2007; Gavini et al., 1991). The strains were also clustered according to their fingerprinting profiles. Genomic DNA from the bacterial strains was obtained using the commercial Genomic DNA Mini Kit (Geneaid). Random amplified polymorphic DNA analysis was performed using the primers 173 and 103 (Sakata et al., 2002); the cycle program reported by Mayer et al. (2007) was used. The primer $(GTG)_5$ was used for a repetitive element sequence-based polymerase chain reaction performed according to Gevers et al. (2001). The similarity between strains was calculated using UPGMA (average from experiments — used primers).

2.5. Sequencing of phylogenetic markers

Based on previous results the strains S7, S10, S13 and S17 were selected for sequencing of phylogenetic markers. Almost complete 16S rRNA gene was amplified, as described previously (Killer et al., 2010). Purified 16S rRNA gene fragments were sequenced by using an automatic genetic analyzer, ABI PRISM 3130xl (Applied Biosystems). Obtained sequences were edited using the Chromas Lite 2.0 software, compared with sequences available at GenBank (Killer et al., 2011) and then deposited at GenBank using Banklt tool (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank). Genes encoding the heat-shock protein 60 kDa (*hsp60*) and elongation factor EF-G (*fusA*) designed as suitable phylogenetic markers for members of the family *Bifidobacteriaceae* (Killer et al., 2013) were amplified in three strains (S 7, S10, S13, and S17) according to Okamoto et al. (2007) and Deletoile et al. (2010), respectively. Obtained sequences of phylogenetic markers were used for identification to species level and phylogenetic studies.

2.6. Phylogenetic studies

Gene sequence similarities of studied bacterial isolates to closest relatives were calculated using jPHYDIT software (Jeon et al., 2005). The 16S rRNA, *hsp60* and *fusA* genes of type strains of species belonging to the genus *Bifidobacterium* and representatives of related genera were obtained from the GenBank database and aligned by ClustalW algorithm within the MEGA 5.05 software. All alignments were then improved by removing hypervariable positions using the Gblocks program under the default conditions (Castresana, 2000). Constructions were carried out using maximum-likelihood method and Jukes–Cantor model in the MEGA 5.05 program. Phylogenetic trees were then visualized using the TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html), FigTree and Inkscape programs.

3. Results and discussion

Bifidobacteria were detected in 8 samples of ovine cheese from different chalets (Table 1); the counts ranged from 10^3 to 10^5 CFU/g cheese. However, the C was found not to be completely selective as propionic bacteria were able to grow. Several isolates of bifidobacteria from each cheese were selected. However, preliminary screening of their morphology and fingerprinting profiles showed that the cheese samples contain identical strains. In total, 8 *Bifidobacterium* isolates (1 isolates per cheese) were selected for detail identification. These isolates were identified according to their morphology, fermentation profiles, MALDI-TOF mass spectrometry (BioTyper log score > 2.3, highly probable species identification) and sequencing as *B. crudilactis* and *B. animalis* subsp. *lactis*. The species *B. crudilactis* was detected in 6 samples, whereas the subspecies *B. animalis* subsp. *lactis* was only detected in 2 samples. The cheese isolates identified as *B. crudilactis* were divided into 2 groups according their fingerprinting profiles. The isolates S10, S11, S13, S15, and S18 were identical, while the isolate S17 shown slightly different fingerprinting profile (Fig. 1). Compared to the type strains, cheese isolates identified as *B. crudilactis* showed slightly different fingerprinting profiles when analyzed by random amplified polymorphic DNA-polymerase chain reaction and repetitive element sequence-based polymerase chain reaction. These differences are also evidenced in the phylogenetic tree reconstructed by maximum-likelihood method showing the position of strains isolated from ovine cheese (Fig. Fig. S1 and Fig. S2). However, the fingerprinting profiles of *B. animalis* subsp. *lactis* obtained with the used primers were identical for the new cheese strains (S1 and S7) and the type strain (Fig. 1). These primers have been successfully used in many studies for strain differentiation of bifidobacteria (Bunesova et al., 2012; Krizova et al., 2008; Sakata et al., 2002). The partial 16S rRNA gene sequences of selected strains S7, S10, S13 and S17 strains are available at the GenBank database under the accession numbers KJ463393, KJ463394, KJ463395 and KJ463396, respectively. The accession numbers of strains S10, S13, S17 and *Bifidobacterium psychraerophilum* DSM 22366^T for partial *hsp60* and *fusA* gene sequences are KJ463397–KJ463404. The strain S7 was identified based on 16S rRNA gene